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Seasonal dominance of CL500-11 bacterioplankton (phylum Chloroflexi) in the oxygenated hypolimnion of Lake Biwa, Japan

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- 1 **Seasonal dominance of CL500-11 bacterioplankton (Phylum Chloroflexi) in the oxygenated**
- 2 **hypolimnion of Lake Biwa, Japan**
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- 12

ABSTRACT

Uncultured bacteria affiliated with the CL500-11 cluster (phylum Chloroflexi) were first reported from the oxygenated hypolimnion of Crater Lake (USA) as a predominant bacterioplankton, although this dominance has not been reported in other environments. In this study, we showed that CL500-11 is also dominant in the oxygenated hypolimnion of Lake Biwa (Japan) and followed its spatiotemporal succession using fluorescent *in situ* hybridization. CL500-11 cells were almost absent (<1% of DAPI-stained cells) at the beginning of the stratification period, dominated (>10% of DAPI-stained cells; maximum = 16.5%) in the hypolimnion during the stratification period, and decreased to below the detection limit with the collapse of the thermocline. This pattern was observed over two annual cycles. A longitudinal assessment also showed that CL500-11 was the dominant bacterium in the hypolimnion over the whole lake but was generally undetectable in the stratified epilimnion. These data suggest that CL500-11 is acclimated to the oxygenated hypolimnion and is a potentially important component of the pelagic biogeochemical cycling of the lake. A comparative analysis of 16S rRNA gene sequences revealed that almost all CL500-11 sequences previously deposited in the database were detected from hypolimnion or holomictic water in deep oxic freshwater lakes, suggesting that the bacteria may form one of the common lineages residing in an aerobic hypolimnetic niche.

INTRODUCTION

Planktonic bacteria are the most abundant and active organisms in the pelagic zones of lakes. The recent development of molecular tools has allowed researchers to identify bacterial communities as assemblages of functionally diverse phylogenetic groups, and current study trends are now directed toward the characterization of each group (Hahn et al., 2005; Newton et al., 2007; Watanabe et al., 2009; Šimek et al., 2010). To understand the basic ecological features of a particular bacterial group, cells specifically detected by fluorescent *in situ* hybridization (FISH) (Pernthaler et al., 2004; Amann and Fuchs, 2008) are directly counted, and their distribution in response to spatial and temporal environmental heterogeneity is assessed (Allgaier and Grossart 2006a; Salcher et al., 2008, 2011b). Some FISH studies have demonstrated cyclic dominances of particular bacterial groups. For instance, it has been reported that Polynucleobacter (Betaproteobacteria) and LD12 clade (Alphaproteobacteria) are dominant, respectively, in the epilimnion in Lake Mondsee (Wu and Hahn, 2006) and in prealpine lakes (Salcher et al., 2011a) during the summer stratification period. FISH studies have further suggested that each bacterial group occupies a particular ecological niche and dominates in response to transitions in niche availability.

In lake ecosystems, the hypolimnion consists of a separated water layer under the thermocline during periods of stratification. In holomictic lakes, hypolimnetic water is mixed with epilimnetic water through winter vertical mixing. In such cases, hypolimnetic bacteria play important roles in the biogeochemical cycling of the lake by mineralizing organic matter and regenerating nutrients that may then be supplied to the epilimnion through winter vertical mixing (Wetzel, 2001). As the amount of oxygen required for hypolimnetic mineralization does not exceed the hypolimnetic oxygen stock during the stratified period, the hypolimnion of oligo- to mesotrophic lakes often remains oxygenated throughout the year (Wetzel, 2001). As the oxygenated hypolimnion consists of an isolated water mass during the stratification period, it may provide a distinct ecological niche to be inhabited by bacterioplankton adapted to this environment. Nevertheless, information on the dominant phylotypes in the oxygenated hypolimnion remains scarce, in contrast to the extensively explored epilimnion.

The CL500-11 cluster of the phylum Chloroflexi was described by Urbach et al. (2001) as a predominant group of bacterioplankton in the oxygenated hypolimnion of the freshwater ultraoligotrophic Crater Lake, USA. Quantitative rRNA-targeted dot-blot hybridization indicated that CL500-11 represented around 50% of the nonplastid bacterial rRNA in the hypolimnion during the summer stratification period (Urbach et al., 2001, 2007), suggesting that the cluster is a potential player in the process of hypolimnetic mineralization. However, CL500-11 dominance has been reported only from Crater Lake to date, and this is the single known case of Chloroflexi dominance in an oxygenated freshwater system. A growing consensus on major bacterial groups in oxygenated freshwaters indicates that the phyla Proteobacteria, Bacteroidetes, and Actinobacteria are generally the predominant groups of bacterioplankton, irrespective of trophic status or local climate (Zwart et al., 2002; Newton et al., 2011). Therefore, Chloroflexi CL500-11 bacteria have been regarded as an unusual phylotype whose ecological characteristics remain obscure because of lack of information.

Here, we report on the identification of a second case of CL500-11 dominance in the oxygenated hypolimnion, in this case, from Lake Biwa, Japan. This discovery supports the idea that the ecological niche of the CL500-11 cluster is not limited to the hypolimnion of Crater Lake but also occurs in general aerobic deep freshwaters. In the present study, we quantitatively followed the vertical, horizontal, and seasonal distributions of the bacterium using catalyzed reporter deposition (CARD)-FISH. Additionally, 16S rRNA gene sequences, affiliated with the CL500-11 clusters that have been deposited in the Genbank/EMBL/DDBJ sequence database, were also examined, and by integrating our novel data with the currently available knowledge on the CL500-11 cluster, we provide new insights into the ecological characteristics of this bacterium.

MATERIALS AND METHODS

Study site and sampling

Lake Biwa is a mesotrophic monomictic lake with a surface area of 674 km², a maximum depth of 104 m, and an estimated water retention time of 5.5 years. The lake has a permanently oxygenated hypolimnion, where the annual minimum dissolved oxygen concentration generally stays above 100

μM (Kim et al., 2006). Spatiotemporal measurements of dissolved and particulate C, N, and P in the lake are available in Kim et al. (2006). We collected water samples from pelagic stations in Lake Biwa (Fig. 1) using a 5-L Niskin-X bottle (General Oceanics, Miami, FL), and determined water temperature using a CTD profiler (SBE-911plus, Sea Bird Electronics, Sealogger, WA). Monthly samples were collected from Sta. 6 (water depth = ca. 73 m) from December 2009 to September 2011 (Fig. 1). Longitudinal samples were collected from Stations 3 through 7 in September 2011 (Fig. 1). The sampling depths were 5 m (epilimnetic water) and 3 to 6 depths between 35 m and 1 m above the bottom surface in 15-m intervals. For the first 3 months of the monitoring, we collected water samples from depths of 5 m, 30 m, 50 m, and the bottom (December 2009) or from the depths of 5 m, 25 m, 50 m, and the bottom (January and February 2010). Immediately after collection, water samples were fixed with glutaraldehyde (1% final concentration) for the enumeration of bacteria and with newly prepared buffered paraformaldehyde (pH 7.4, 1% final concentration) for use in CARD-FISH. All fixed samples were kept under cool conditions until further processing.

CARD-FISH

To detect the CL500-11 cluster in Crater Lake, Urbach et al. (2001) designed an oligonucleotide probe “CLGNS-584” for hybridization to membrane-blotted environmental rRNA. We used this same specific sequence to construct an HRP-labeled oligonucleotide probe (5'-GCCGACTTGCCCAACCTC-3') (Thermo Fisher Scientific, MA). However, the CLGNS-584 probe alone did not produce sufficient signal intensity to clearly distinguish positive cells from the background. This is presumably because the target site of CLGNS-584 (*Escherichia coli* position 585-602) is located in helix 22 of the 16S rRNA structure, where accessibility to the probe is low (Behrens et al. 2003). To improve the accessibility of CLGNS-584, we designed a helper oligonucleotide, which is an unlabeled oligonucleotide that binds at a location adjacent to the target site of the labeled probe and presumably enhances probe accessibility to the site by opening the secondary structure of the rRNA (Fuchs et al., 2000). The designed helper oligonucleotide, CLGNS-567h (5' - CTACACGCCCTTTACGCC - 3') targeted *E. coli* positions 567–584. According to the Probe Match tool of the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu>), every

16S rRNA sequence targeted by CLGNS-584 had a completely complementary sequence to CLGNS-567h.

Total bacterial abundance was determined by the 4',6-diamidino-2-phenylindole (DAPI) method (Porter and Feig 1980), with at least 900 bacterial cells enumerated in each sample. The CARD-FISH procedure was performed as described by Pernthaler et al. (2004), with some modifications. Briefly, within 6 h of fixation, 3 mL of the samples were filtered through a 0.2- μ m polycarbonate filter. The collected cells were then coated with 0.1% (w/v) low-gelling-point agarose and permeabilized with lysozyme solution [0.05 M EDTA, 0.1 M Tris-HCl pH 8.0, 10 mg mL⁻¹ lysozyme (MP Biomedicals, Santa Ana, CA)] for 40 min at 37°C. The filter was cut into 6 pieces and soaked with 300 μ L of hybridization buffer [900 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.02% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) blocking reagent, 10% (w/v) dextran sulfate, 20% (v/v) formamide] containing 0.5 μ g mL⁻¹ of probe CLGNS-584 and 0.1 μ g mL⁻¹ of helper CLGNS-567h. The optimum formamide concentration was determined by testing a series of concentrations (0–50% at 10% intervals) to produce maximum signal intensity. The hybridization reaction was conducted in a 24-well microplate overnight at 46°C with mild agitation (10 rpm). After hybridization, the filter pieces were washed with 1 \times PBS twice for 10 min at 46°C. Stringent washing was omitted, as Wendeborg (2010) reported that it did not make a significant difference to CARD-FISH results, as CARD-FISH works with lower concentrations of the probe than does FISH using fluorochrome-labeled probes. We also tested the effect of the washing step and found that it did not affect our results. Fluorescein-labeled tyramide signal amplification was performed using the TSA Fluorescein System (NEL701001KT, Perkin Elmer, Waltham, MA). The filter pieces were then soaked in 30 μ L of amplification mixture [1 \times amplification diluent:40% (w/v) dextran sulfate:fluorescein-tyramide reagent = 25:25:1] in a 24-well microplate and incubated in the dark for 45 min at 46 °C before being mounted on a glass slide with an anti-fading reagent [Citifluor (Citifluor, UK):Vectashield (Vector Laboratories, Burlingame, CA) = 4:1] containing 1 μ g mL⁻¹ of DAPI. For each sample, at least 1000 DAPI-positive cells and the corresponding FISH-positive cells were enumerated with UV and blue excitation, respectively, under an epifluorescence microscope.

The CLGNS-584 positive cells were easily identified, because they showed bright fluorescence and a distinctive crescent shape. Negative control with the nonsense probe NON338 (Wallner et al., 1993) demonstrated that no false positive or autofluorescent objects were confused with the enumerated CLGNS-584 positive cells.

Cloning and phylogenetic analyses

To identify partial 16S rRNA gene sequences of CL500-11 bacteria, we performed a clone library analysis using a sample collected at a depth of 65 m at Sta. 6 (Fig. 1) in December 2010. Twenty-five milliliters of non-fixed sample were filtered through a 0.2- μ m polycarbonate filter. DNA was extracted from the seston collected on the filter using cetyltrimethylammonium bromide, chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1) according to Wilson (2001). The extracted DNA was resuspended in 40 μ L of TE buffer. We used the universal primers 341f (Muyzer et al., 1993) and 907r (Muyzer et al., 1995) to amplify the eubacterial 16S rRNA gene. PCR amplification was performed in a 25- μ L reaction mixture with a Blend Taq PCR kit (Toyobo, Japan) using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The PCR was performed under the following conditions: after 9-min preincubation at 94°C, 29 cycles at 94°C for 1 min, followed by annealing temperature for 1 min and 72°C for 3 min. In the first 19 cycles, the annealing temperature was reduced by 1°C after every two cycles from 64°C in the first cycle to 55°C in the nineteenth. During the last 10 cycles, the annealing temperature was 55°C, followed by a final extension at 72°C for 9 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, the Netherlands) and cloned using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI) according to the manufacturer's instructions. We picked 100 positive colonies and amplified the inserted DNA fragments with T7 and U19 primers. The amplified fragments were verified by 2% agarose gel electrophoresis, and the DNA from the colonies, with an insert of the correct size, was sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) and an ABI3100-Avant Capillary Auto Sequencer.

The acquired partial 16S rRNA gene sequence of CL500-11 and the corresponding part (341–926 in *E. coli* numbering) of the related reference sequences retrieved by BLAST search

(Altschul et al., 1997) were examined for phylogenetic analysis. The sequences were aligned using CLUSTALW and a neighbor-joining phylogenetic tree was then constructed using MEGA version 5.05 software (Tamura et al., 2011). Only sequences covering >95% of the target segment (341–926 in *E. coli* numbering) were analyzed and included in the tree.

Based on the constructed tree, related partial sequences covering <95% of the target segment were retrieved manually using BLAST search and the SeqMatch tool of the RDP. Sequences showing >98% similarity with the CL500-11 original full sequences (Accession number: AF316759) (Urbach et al., 2001) were considered to be affiliated with the CL500-11 cluster, and the resulting information was summarized.

RESULTS

Temperature profile and bacterial abundance

The obtained temperature profile indicated that Lake Biwa was vertically mixed from January to March and thermally stratified from April to December (Fig. 2A). The water temperature ranged between 7.2°C and 30.2°C in the epilimnion (5 m) and was <10°C in the hypolimnion. In February 2010 and February 2011, differences in temperature between bottom and surface waters were <0.1°C, indicating that the lake was holomictic during the mixing periods of those 2 years.

During the stratified period, total bacterial abundance varied from 1.7×10^6 to 10.9×10^6 cells mL⁻¹ in the epilimnion and 0.7×10^6 to 1.8×10^6 cells mL⁻¹ in the hypolimnion. Exceptionally, 2.2×10^6 cell mL⁻¹ was recorded from the bottom of the lake in July 2011. During the mixing period, bacterial abundance ranged from 1.3×10^6 to 3.0×10^6 cells mL⁻¹ (Fig. 2B).

Spatiotemporal distribution of CL500-11 bacteria

CARD-FISH performed in conjunction with the helper oligonucleotide technique resulted in high signal intensity of positive cells; the CL500-11 cells thus detected exhibited curved-rod morphology with 1–2 µm length and 0.2–0.3 µm width (Fig. 3). Our monthly monitoring revealed an annual cyclic vertical distribution of CL500-11 (Fig. 4A, B). The percentage of CL500-11 to DAPI-stained

cells at 50-m depth during the stratified period in 2010 increased from below the detection limit in April to 12.3% (1.4×10^5 cells mL^{-1} in abundance) in December. Similar trends were found at 65 m and in the bottom water, with percentages of 12.4% and 12.6% (1.1×10^5 and 1.3×10^5 cell mL^{-1}) in December 2010, respectively. At a depth of 35 m, the percentage also started to increase in April, reaching 8.8% (1.1×10^5 cell mL^{-1}) in September. Thereafter it decreased, presumably due to the collapse of the thermocline (Fig. 2A). In contrast, CL500-11 cells were generally undetectable in the epilimnion (5 m) during the stratified period in 2010 (Fig. 4A, B). Subsequent holomixis, however, distributed the CL500-11 cells across the water layers. The percentage of CL500-11 cells to DAPI-stained cells varied from 2.1% to 4.9% (0.4×10^5 to 1.2×10^5 cells mL^{-1}) at the studied depths in January 2011 and then decreased to 0.0–0.8% (0.0×10^5 to 0.1×10^5 cells mL^{-1}) in April 2011 (Fig. 4A, B).

The longitudinal distribution of the percentage of CL500-11 bacteria in relation to DAPI-stained cells in the hypolimnion (sampling depth, 35–103 m) ranged between 10.0% and 16.5% (1.0×10^5 to 1.9×10^5 cell mL^{-1}) in September 2011 (Fig. 4C, D). The percentage of CL500-11 bacteria in the epilimnion (sampling depth, 5 m) was below the detection limit at all stations.

Comparative analysis of 16S rRNA gene sequences

We obtained 83 sequences with clone library analysis. Eleven sequences were identical and showed 99.3% similarity to the original CL500-11 sequence (Accession number: AF316759) (Urbach et al., 2001). The acquired partial 16S rRNA gene sequence of the CL500-11 cluster, LB65D-54, was deposited in the DNA Data Bank of Japan (accession number; AB686531).

A phylogenetic tree indicated that the CL500-11 cluster is a member of the class Anaerolineae of the phylum Chloroflexi (Fig. 5). Pairwise similarities among the CL500-11 sequences exceeded 97.3% in all combinations. Clone 52-3-31, which was the closest clone located outside the CL500-11 cluster, showed 92.5% similarity to the clone LB65D-54 (Fig. 5). This sequence and four other sequences originating from deep oceans formed a distinct cluster (“deep-ocean cluster” in Fig. 5). Other Anaerolineae clones were derived from a variety of

environments, including sediment, soil, sludge, symbiotic systems, and surface planktonic habitats (Fig. 5). Note that the analysis was conducted using a 16S rRNA partial sequence (341–926 in *E. coli* numbering) and that the resulting tree was almost consistent with a tree constructed from full-length analysis (Yamada and Sekiguchi, 2009).

Including partial sequences that were not assigned in the tree, we found 24 sequences affiliated with the CL500-11 cluster (Table 1). The sequences originated exclusively from freshwater environments. Four clones were reported from the hypolimnetic water of Crater Lake during the summer stratified period (Urbach et al., 2001). Four additional clones were retrieved from hypolimnetic samples collected from three deep subalpine lakes during their stratified periods, whereas six clones were collected from holomictic water samples (Humbert et al. 2009). In Yellowstone Lake, two studies individually investigated the bacterial community in the bottom waters near hydrothermal vents and found CL500-11 sequences (Clingenpeel et al., 2011; Yang et al., 2011). The other CL500-11 bacteria clones were reported from metalimnion (Van den Wyngaert et al., 2011), hypolimnion (Han et al., unpublished sequence), and holomictic (Allgaier and Grossart, 2006b) waters sampled from deep oxic lakes, with the exception of two clones obtained from the tap water of a Norwegian hospital (Rudi et al., 2009).

DISCUSSION

Ecological niche of the CL500-11 cluster

Using FISH counts, the present study first quantitatively followed annual succession in the CL500-11 population. The data indicate that CL500-11 bacteria are adapted to the stratified hypolimnion of Lake Biwa. Consistently, Urbach et al., (2007) have also reported high contributions of CL500-11 in the bacterial community of the oxygenated hypolimnion of Crater Lake during three consecutive summers. Moreover, almost all previously reported CL500-11 sequences were obtained from cells derived from oligo- to mesotrophic and holomictic lakes that had year-round oxygenated hypolimnion waters, with none derived from epilimnetic water during the stratification period (Table 1). Taken together, these findings suggest that CL500-11 bacteria may be generally distributed in the

oxygenated hypolimnion of freshwater lakes. However, it is still unclear whether CL500-11 forms a dominant cluster in lakes other than Crater Lake and Lake Biwa, as these bacteria have not yet been quantitatively evaluated in other lakes. It should be noted that extensive clone library analyses of the bacterial community of the oxygenated hypolimnion of Lake Baikal did not detect the presence of the CL500-11 sequence (Glöckner et al., 2000; Bel'kova et al., 2003). This suggests the existence of unknown environmental factors crucial for CL500-11 survival, although an oxygenated hypolimnion may be one of the necessary conditions. Ultraoligotrophy and growth limitation by N and trace metals, both of which are traits of Crater Lake water (Urbach et al., 2001, 2007), are not likely factors influencing CL500-11 occurrence because Lake Biwa is a mesotrophic, P-limited lake (Kim et al., 2006). Dissolved oxygen concentration in the hypolimnion is also unlikely to be a key factor, as it decreases by less than 150 μM in the late stratification period in Lake Biwa (Kim et al., 2006) and is generally $>290 \mu\text{M}$ throughout the year in Crater Lake (McManus et al., 1996). As CL500-11 dominance has been reported only from these two lakes, the available data are not sufficient to identify the factors that potentially trigger the dominance of the bacteria. Quantitative information and comparative analyses of CL500-11 occurrences in lakes worldwide are needed in future studies.

Ecological diversification of CL500-11 within the phylum Chloroflexi

To date, only two phylogenetic groups of Chloroflexi are known to be predominant in aerobic planktonic habitats: the freshwater CL500-11 cluster and the marine SAR202 cluster (Giovannoni et al., 1996; Morris et al., 2004). The SAR202 cluster belongs to “subphylum IV” of the Chloroflexi phylum and is phylogenetically distant from the CL500-11 cluster in “subphylum I” (Yamada and Sekiguchi, 2009). Nonetheless, interestingly, the SAR202 cluster also dominates in deep-water environments, comprising 1.5 to 15% of all prokaryotic cells in deep layers of both the Atlantic and Pacific Oceans (Morris et al., 2004; Varela et al., 2008; Schattenhofer et al., 2009). It is also remarkable that the CL500-9 cluster, which is a subgroup of SAR202, was found in the bacterial community of the hypolimnion of Crater Lake, albeit not in high abundance (Urbach et al., 2001). Currently, very little is known about the eco-physiology of SAR202 bacteria. Varela et al. (2008) demonstrated that SAR202 bacteria preferably utilize L-aspartic acid (Asp) over D-Asp, although

the percentages of bacteria taking up L-Asp decrease with depth. This result suggests that SAR202 may efficiently uptake available substrates in deep marine environments. Further studies of SAR202 bacteria are needed to elucidate its ecology in oxygenated deep-water environments, and this information may be applicable to future studies of CL500-11 bacteria.

It is also noteworthy that the CL500-11 cluster is phylogenetically grouped with a cluster derived exclusively from marine oxygenated deep waters (“deep-ocean cluster” in Fig. 5). The characteristics of the deep-ocean cluster are currently completely unknown because all of the reported sequences isolated from these waters have been documented as minor eccentric groups found in total bacterial community analyses (Pham et al., 2008; Galand et al., 2010; Redmond and Valentine, 2011; Swan et al., 2011). For instance, in a study of the deep Arctic Ocean, only 5% of the bacterial pyrosequencing reads belonged to phylum Chloroflexi, and the contribution of the deep-ocean cluster within the phylum was small (Galand et al., 2010). In a fosmid-based metagenomic analysis of the bacterial community at depths of 500 m and 770 m at the North Pacific Time-series Station ALOHA, sequences affiliated with the deep-ocean cluster accounted for less than 1% of the clones collected from both depths (Pham et al., 2008). Thus, it is likely that the deep-ocean cluster accounts for only a minor fraction of the deep marine bacterial community. Nonetheless, the striking similarities in habitats of the two sister clusters implies that they may have common eco-physiological traits. More ecological information on the deep ocean cluster should be collected for comparison with the CL500-11 cluster.

Clearly, it may not be appropriate to conclude the physiology of the CL500-11 cluster from that of its phylogenetic relatives. To date, only 6 species have been isolated and characterized in the class Anaerolineae, (Fig. 5), all of which show anaerobic growth, multicellular filamentous morphology, and mesophilic or moderately thermophilic preferences (Yamada and Sekiguchi, 2009). CL500-11 bacteria lack these characteristics. Additionally, the class contains environmental sequences derived from a broad range of habitats (Fig. 5). The class also includes sequences from the surface planktonic habitat (Fig. 5), although individuals are less abundant in this habitat (Shaw et al., 2008; Van den Wyngaert et al., 2011). Thus, the class Anaerolineae contains a variety of bacteria,

and its common characteristics are still unknown.

Ecological roles of the CL500-11 cluster

Our longitudinal monitoring demonstrated that CL500-11 bacteria comprised more than 10% of the total bacterial population in the hypolimnion of Lake Biwa during the summer-stratified period (Fig. 4C, D). CL500-11 bacteria likely account for an even larger proportion of the community in terms of biomass because of their relatively large cell size (Fig. 3). This substantial abundance suggests that CL500-11 bacteria play a potentially important role in pelagic biogeochemical cycling in the lake. It is conceivable that CL500-11 bacteria utilize some dissolved substances for their growth, because almost all of the CL500-11 bacteria in this study were found to be free-living, and a subset were undergoing division at the time of analysis (arrows in Fig. 3B). Following a carbon stable isotope ratio analysis of the spatiotemporal dynamics of dissolved organic carbon (DOC) in Lake Biwa, Maki et al. (2010) suggested that a fraction of the DOC derived from surface photosynthetic production escapes from rapid degradation and accumulates in the epilimnion (semi-labile DOC), before being transferred to the hypolimnion by winter mixing and subsequently degraded over months during the stratified period. Thus, it may be that CL500-11 bacteria play a pivotal role in the recycling of the semi-labile fraction of DOC in the hypolimnion.

To clarify the substrate availability of CL500-11 bacteria, cultivation of an isolated strain will be crucial. However, a thorough effort by Page et al. (2004) to isolate previously uncultivated bacterial strains from Crater Lake using a high-throughput cultivation method (Connon et al., 2002), was unsuccessful in isolating CL500-11. This difficulty in isolation evokes another possibility with regard to CL500-11 eco-physiology: mutualistic growth with other organisms. To support this, Urbach et al. (2007) have previously suggested that the dominance of CL500-11 in the hypolimnion in Crater Lake is related to the occurrence of marine Group I Crenarchaeota, another predominant phylotype in the hypolimnion. Callieri et al. (2009) also reported that the proportion of Crenarchaeota to the total prokaryotic community in a deep oxic subalpine lake increased along with depth, although no information has been provided on the hypolimnetic eubacterial composition of the lake. These results imply that it would be worthwhile to investigate archaeal distribution and its

relationship to CL500-11 succession in Lake Biwa.

Conclusions

In this study, CARD-FISH revealed a seasonal dominance of CL500-11 bacteria in the oxygenated hypolimnion of Lake Biwa, suggesting that CL500-11 is likely an important component of pelagic biogeochemical cycling in the lake. A search of previously deposited CL500-11 sequences in clone libraries suggested that the bacteria might be ubiquitously distributed in oxygenated hypolimnion waters. Therefore, exploration of the bacterial community composition in oxic lakes worldwide and isolation of these bacteria would further elucidate CL500-11 eco-physiology.

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Sampling site	Country	Mean (max.) depth (m)	Area (km ²)	Trophic status	Stratified period	Year-round oxygenated hypolimnion	Sampling depth (m)	Sampling month ^a	No. of clones	Accession number	Reference
Lake Biwa	Japan	41 (104)	670	Mesotrophic	Apr. – Dec.	yes	65	Dec. (s)	1	AB686531	This study
Crater Lake	USA	350 (594)	53	Oligotrophic	May – Nov.	yes	120	Aug. (s)	1	AF316758	Urbach et al., 2001
							500	Aug. (s)	3	AF316759–AF316761	Urbach et al., 2001
Lake Annecy	France	42 (65)	28	Oligotrophic	May – Nov.	yes	2	Jan. (m)	1	AJ965812	Humbert et al., 2009
							50	Jan. (m)	1	AJ965898	Humbert et al., 2009
							50	Aug. (s)	2	AJ965858, AJ965870	Humbert et al., 2009
Lake Bourget	France	85 (145)	45	Mesotrophic	Apr. – Nov.	yes	50	Apr. (s)	1	AJ966070	Humbert et al., 2009
Lake Geneva	France /Switzerland	154 (310)	580	Mesotrophic	Apr. – Nov.	yes	2	Jan. (m)	4	AJ966118,AJ966120, AJ966121,AJ966124	Humbert et al., 2009
							50	Apr. (s)	1	AJ966228	Humbert et al., 2009
Lake Zurich	Switzerland	49 (143)	89	Mesotrophic	May – Nov.	yes	15	Oct. (s)	2	FN668367, FN668368	Van den Wyngaert et al., 2011
Lake Breiter Luzin	Germany	22 (59)	3.6	Mesotrophic	Jun. – Oct.	yes	0–10	Nov. (m)	1	DQ501287	Allgaier and Grossart, 2006b
Lake Soyang	South Korea	42 (110)	44	Mesotrophic	May – Dec.	yes	80	unknown	1	AF107533	Han et al. (unpublished)
Yellowstone Lake	USA	42 (120)	53	Oligotrophic	May – Nov.	yes	21.7 ^b	Jul (s)	1	HM446117	Yang et al., 2011
							52.4 ^b	Jul (s)	1	HM446145	Yang et al., 2012
							unknown ^c	unknown	1	HM856384	Clingenpeel et al., 2011
Tap water	Norway	n/a	n/a	n/a	n/a	n/a	n/a	n/a	2	GQ165424, GQ165425	Rudi et al., 2009

Fig. 1 Lake Biwa sampling stations. Depths are shown in meters.

Fig. 2 (A) Water temperature and (B) abundances of total bacterial (DAPI-stained) cells at the monthly monitoring station (Sta. 6) during the study period. Dots indicate sampling points.

Fig. 3 CARD-FISH images of CL500-11 cells. An identical microscopic field of (A) DAPI-stained cells and (B) CL500-11 bacteria stained by CARD-FISH with a combination of probe CLGNS-584 and helper CLGNS-567h. The sample was taken at Sta. 4 at a depth of 50 m in September 2011. Arrows indicate dividing cells. Images were acquired using a CCD camera (Hamamatsu ORCA-ER, Japan) attached to a microscope with a fixed exposure time (22.0 ms). The scale indicates 10 μ m.

Fig. 4 The percentage of CL500-11 to DAPI-stained cells and CL500-11 abundance (A and B, respectively), determined by monthly monitoring at Sta. 6 and by longitudinal monitoring in September 2011 (C and D, respectively). Dots indicate sampling points.

Fig. 5 Phylogenetic tree of the CL500-11 cluster and representatives of the class Anaerolineae, derived from partial sequences of 16S rRNA gene (*E. coli* position 341-926). The sequence deposited in the present study (LB65D-54) is shaded grey. Cultivated strains are shown in bold, whereas uncultivated gene clones are labeled with the environments from which they originated. Planktonic gene clones are underlined. Nodes supported by bootstrap value of >50% are indicated. The scale bar represents five nucleotide substitutions per 100 nucleotides.

Table 1 Details of the lakes from which the 24 database clones of the CL500-11 cluster originated.

a. (s): stratified period; (m): mixing period.

b. Hydrothermal vent water.

c. Samples were obtained from the near-surface photic zone or hydrothermal vents.

Fig. 1

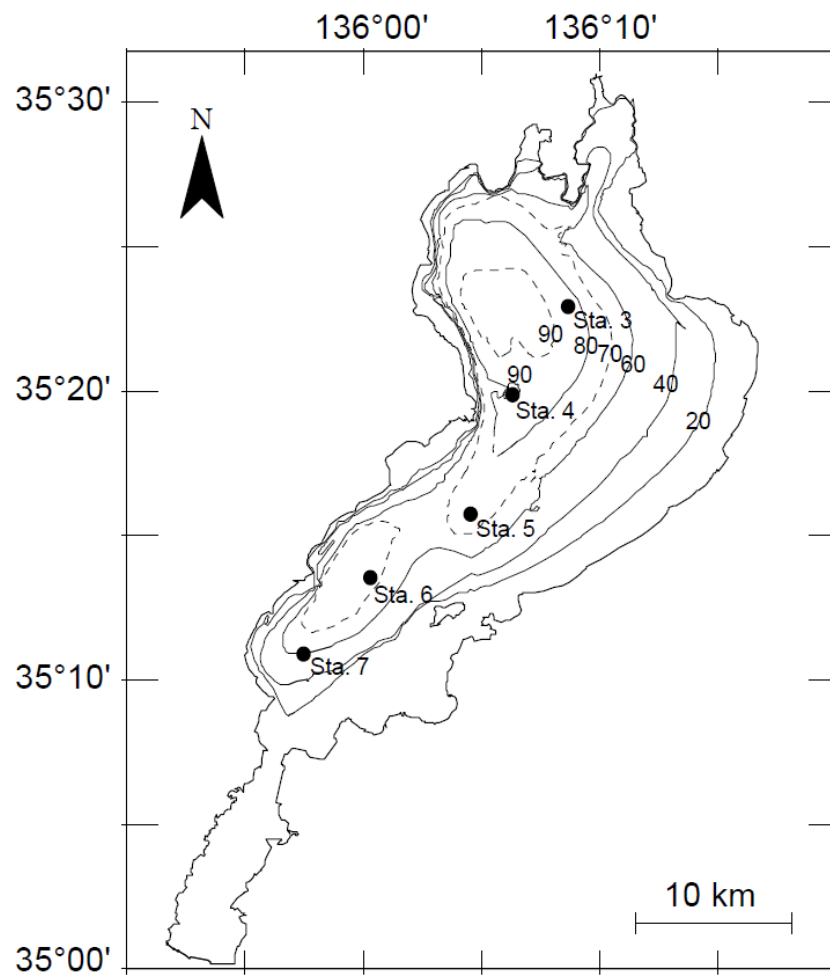


Fig. 3

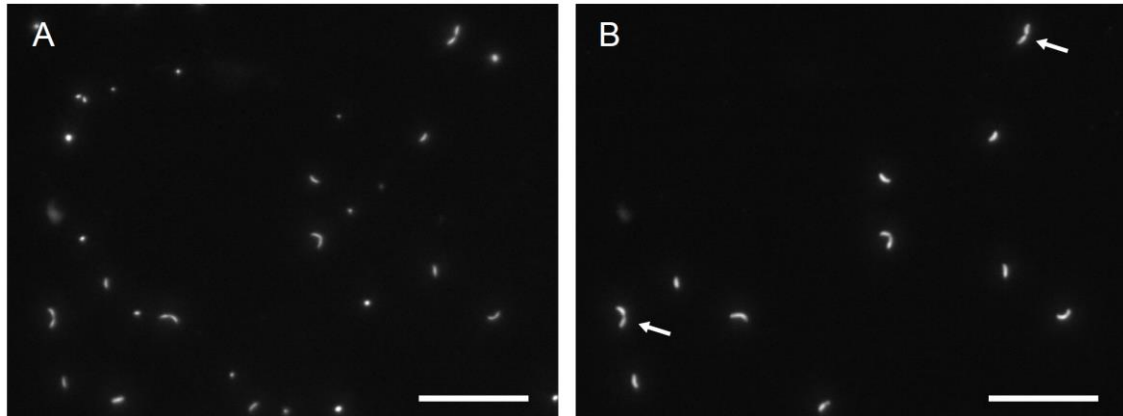


Fig. 4

